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Genes from plasmid pKM101 in *Haemophilus influenzae*: Separation of functions of *mucA* and *mucB*

(UV mutability/postreplication repair/Weigle reactivation)

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ABSTRACT **_Haemophilus influenzae_, normally not mutable by UV, became UV mutable with a recombinant plasmid insertion. A 7.8-kilobase-pair (kbp) fragment of the plasmid pKM101 containing the *mucA* and *mucB* genes was ligated to the shuttle vector pDM2, and a Rec$^-$ strain of *H. influenzae* was transformed with the ligated mixture. All of the transformants, unlike the parent Rec$^-$ strain, were resistant to UV, could carry out postreplication repair and Weigle reactivation, showed greatly increased spontaneous mutation, and contained a plasmid carrying an insert of only 1.2 rather than 7.8 kbp. This plasmid in a *umuC* mutant strain of *Escherichia coli* complemented a pKM101 derivative lacking *mucA* function but with an intact *mucB* gene, although there was no complementation with a *mucA*$^+$ *mucB*$^-$ plasmid, suggesting that the newly constructed plasmid coded for the *mucA* protein; this is in accord with the restriction analysis and hybridization between the plasmid and a probe containing all of the *mucA* gene but only a small fraction of *mucB*. When one of the *H. influenzae* Rec$^-$ transformants lost the plasmid, the resistance to UV was retained but the high spontaneous mutation and UV mutability were not. The fact that there was hybridization between the chromosome of the "cured" strain and a probe containing both *muc* genes but none when almost no *mucB* was present suggested that at least part of the *mucB* gene had been integrated into the Rec$^-$ chromosome. Five different postreplication repair-proficient strains became UV mutable and had high spontaneous mutation rates caused by the putative *mucA* plasmid, indicating that these strains already possessed a chromosomal equivalent of the *mucB* gene.**

The plasmid pKM101 [35.4 kilobase pairs (kbp)] enhances the mutability of *Escherichia coli* and *Salmonella typhimurium* cells by UV and some chemicals (1). The plasmid also increases cellular and phage survival after UV-irradiation as well as Weigle reactivation and Weigle mutagenesis (2). Evidence has been presented that pKM101 exerts its effects not by making the SOS repair system of the cell constitutive but by contributing to part of this system (3). Mutations in the *umuDC* locus of *E. coli* render the cell immutable by UV or 4-nitroquinoline-1-oxide (4). This locus consists of two adjacent genes, *umuD* and *umuC*, which code for 18- and 46-kDa proteins, respectively (5). The plasmid pKM101 suppresses the immutability of the *umuD* or *umuC* mutants (2). An approximately 2-kbp region of the plasmid is responsible for these effects; this region contains two genes, *mucA* and *mucB*, coding respectively for 16- and 45-kDa proteins that are very similar to those of *umuD* and *umuC* (6).

Because of the inability of *Haemophilus influenzae* to be mutagenized by UV and chemicals such as methyl methanesulfonate and mitomycin C (7, 8), it is possible that this bacterium is the equivalent of an *umuD* or *umuC* mutant strain of *E. coli* (9, 10). To investigate this possibility, we attempted unsuccessfully to grow pKM101 in *H. influenzae*. The alternative approach, which was successful, was to put one or both *muc* genes from pKM101 into a plasmid that would be accepted by *H. influenzae*.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, Phage, and Media. The *H. influenzae* strains used were wild types BC200 and Rd, derivative recombination-defective BC200rec1 and *rec2* mutant strains, and excision-defective *uvr1* and *uvr2* mutant strains (11–13). *S. typhimurium* TA100 (14), which carries the plasmid pKM101 (15), was kindly donated by Sanford Lacks. *E. coli* strain TK610 (4) was obtained from the *E. coli* genetic stock center at Yale University. The cloning vector pDM2 was constructed in this laboratory (16) and confers resistance to 4 μg of chloramphenicol and 5 μg of ampicillin per ml. TK610(pSE200), TK610(pGW1700), TK610(pGW1700-mucA123:Tn1000), and TK610(pGW1700mucB58:Tn1000) (17, 18) from Graham Walker's laboratory were kindly sent by David Sobell. The lysogenic phage HP1*c*1 has been described (19). *H. influenzae* was grown and diluted for plating as before (10). TA100 and TK610 were grown in nutrient broth (Difco). M9 agar medium (18) supplemented with the nutritional requirements of TK610 (threonine, leucine, proline, isoleucine, valine, and thiamine) and 0.02 mM histidine was used to assay for TK610 His$^+$ revertants. Cleared lysates and purified plasmids of *H. influenzae* were prepared as described (20), and pKM101 was purified as described by Maniatis *et al.* (21).

Transformation. Transformation of *H. influenzae* was carried out with the MIV method (22), and *E. coli* was transformed by the $CaCl_2$ method (23).

Gel Electrophoresis. This was carried out in a 1% agarose gel horizontal system (24). Sizing of the fragments was by comparison of mobilities with those of *Hpa* I and *Dpn* II fragments of phage T7 donated by F. W. Studier.

Cloning of Part of pKM101 in the Plasmid Vector pDM2. pKM101 was digested with *Hpa* I (Bethesda Research Laboratories), and a 7.8-kbp fragment containing the *mucA* and *mucB* genes (25) was isolated by electrophoresis of the restriction digest on a low-melting-point agarose gel at 60 V for 3 to 4 hr. The fragment was cut from the gel, the agarose was melted at 65°C, and the DNA was extracted twice with phenol at 37°C, followed by precipitation with ethanol. The cloning vector was digested with *Pst* I (BRL), which cuts in the β-lactamase gene (16), and was treated with S1 nuclease to remove the sticky ends and to prepare the linearized vector for ligation with the blunt-ended fragment from pKM101 and then with alkaline phosphatase (23) to prevent self-ligation of the vector. Ligation with T4 DNA ligase (BRL) was for 4 hr

Abbreviation: kbp, kilobase pair(s).

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at 12°C, followed by 4°C overnight. The ligation mixture was used to transform BC200rec1.

Complementation of *mucA* or *mucB*. The newly constructed pMuc plasmid was transformed into a TK610 strain containing either the plasmid pGW1700mucB58:Tn1000 or pGW1700mucA123:Tn1000 and was grown in chloramphenicol and tetracycline to maintain both plasmids before UV irradiation and measurement of survival and mutation. These strains were compared with the plasmidless TK610.

UV Irradiation. *H. influenzae* cells were grown to an OD_{675} of 0.5 (around 10^9 cells per ml), diluted by a factor of 10, and irradiated as before (26). *E. coli* was grown to an OD_{675} of 0.3 and then was diluted by a factor of 2.4 for irradiation.

Spontaneous and UV-Induced Mutation. Mutation of *H. influenzae* to novobiocin (2 μg/ml)- or kanamycin (4.5 μg/ml)-resistance was measured on agar plates containing the appropriate amount of antibiotic. Cells were plated after UV irradiation without antibiotics and then were allowed to express the mutation for 90 min at 37°C before the plates were layered with agar-containing antibiotic. Mutation of *E. coli* was measured as described by Perry and Walker (17).

Weigle Reactivation. The method has been described (27).

Postreplication Repair. The molecular weight of DNA pulse-labeled after UV irradiation was measured as before on a single-strand basis by centrifugation in alkaline sucrose (28).

Hybridization. The method of Southern (29) was used as modified by F. W. Studier (unpublished data). Nick translation was as described by Maniatis *et al.* (21).

RESULTS

Cloning of the 7.8-kbp Fragment of pKM101 in pDM2. Seventeen transformants of BC200rec1 were obtained that were chloramphenicol resistant and ampicillin sensitive, indicating that the cells contained a plasmid with an insert. Because of the method used for transformation by the ligated mixture, in which the exposed cells were plated before there was time for cell duplication, there was no possibility that any of the transformants were siblings. In all of the transformants, the insert was about 1.0 kbp rather than the complete 7.8-kbp fragment of pKM101 ligated to pDM2. Restriction analysis of one of the recombinant plasmids (Fig. 1) showed that the insert was one continuous piece of DNA and that the plasmid was digested by *Bam*HI, *Pvu* I, *Pvu* II, and *Sma* I but that there were no sites for *Acc* I and *Bgl* II, and there was no *Sma* I site within the insert. The data ruled out the presence of an intact *mucB* gene on the plasmid, in accord with the published restriction map of pKM101 (25) and the known size of the mucB protein (17). The recombinant plasmids were called pMuc.

UV Resistance, Spontaneous Mutation, and UV-Induced Mutation of pMuc Transformants. Fig. 2 shows a comparison of survival curves of BC200rec1 and one of the transformants containing a recombinant plasmid. The strain containing pMuc was considerably more UV resistant and was approximately as UV resistant as the wild type (data not shown). All of the other 16 BC200rec1 transformants obtained from the ligation mixture were also UV resistant. The recombination defect was still present in the pMuc transformants. The parent plasmid, pDM2, had no effect on the UV resistance of the BC200rec1 strain (data not shown).

All of the BC200rec1 transformants showed a frequency of spontaneous mutation to novobiocin resistance of 10^{-4} to 10^{-3}, whereas the untransformed BC200rec1 frequency was 10^{-8} to 10^{-7}. When the plasmid from BC200rec1 was transformed into a wild-type strain, there was no effect on UV resistance, but the frequency of spontaneous mutation to novobiocin resistance was again greatly increased. Fig. 3 shows that there was also UV-induced mutation to novobiocin resistance, in spite of the already high level caused by the presence of the plasmid without UV. Similar results were obtained with pMuc in a number of other strains, all proficient in postreplication repair: wild-type Rd and *rec2*, *uvr1*, and *uvr2* mutant strains. The BC200rec1 transformants also showed UV-induced mutation, with increases in frequency of up to a factor of 5. The wild-type strains containing pMuc had an increased frequency of spontaneous mutation to kanamycin resistance (from 10^{-7}–10^{-6} to 10^{-5}–10^{-4}), and

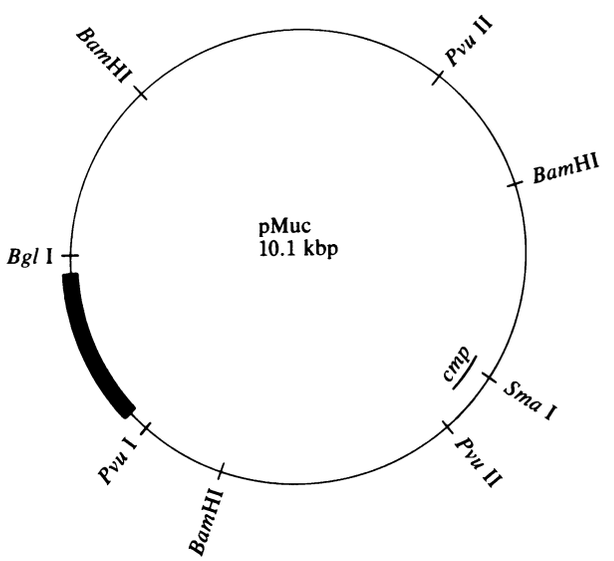

FIG. 1. Restriction map of pDM2 containing an insert from pKM101 (heavy line).

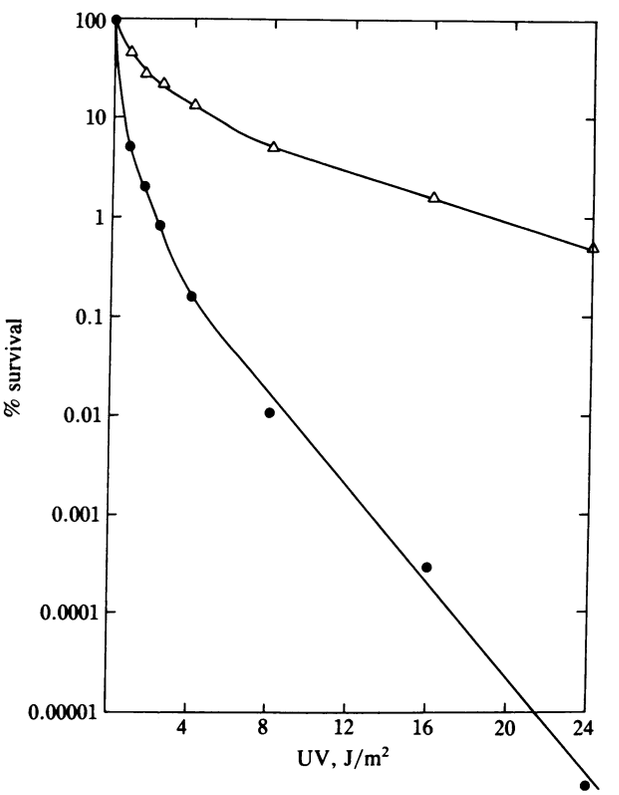

FIG. 2. UV dose effect curves of BC200rec1 with (△) and without (●) pMuc.

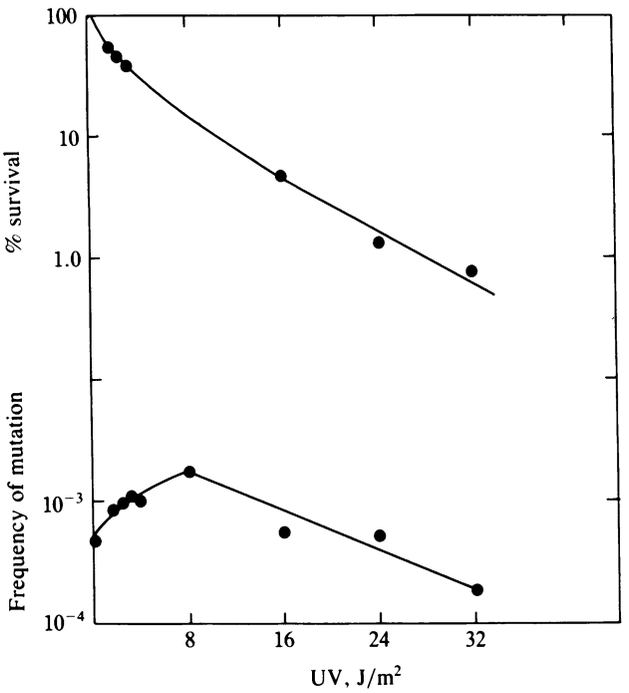

FIG. 3. UV dose–effect curves of BC200(pMuc); mutation to novobiocin resistance (number of mutants per survivor) (*Lower*) and survival (*Upper*). The mutation to novobiocin resistance in cells without pMuc was undetectable under the same conditions as used for BC200(pMuc).

there was a 2-fold increase in mutation to kanamycin resistance resulting from UV.

Postreplication Repair of BC200rec1(pMuc). Table 1 shows the results of measurement of single-strand-based molecular weights of pulse-labeled DNA in irradiated and control cells, incubated various times in nonradioactive medium following the pulse. The data indicate that, in the cells containing pMuc, the single-strand-based molecular weight of DNA synthesized after UV-irradiation returned to a value close to that of the control (unirradiated DNA) value upon incubation after the pulse, but there was little or no change in the DNA of cells without pMuc, in accordance with previous observations with a *rec1* mutant strain (28).

Weigle Reactivation of BC200rec1(pMuc). Fig. 4 shows that there was a substantial increase in survival of the UV-irradiated phage HP1*c*1 in BC200rec1(pMuc) cells given various low doses of UV, but there was little or no increase in BC200rec1 without the plasmid, a result also obtained previously (27).

Properties of a BC200rec1(pMuc) Transformant and Other Strains Carrying pMuc After Loss of the Plasmid. The BC200rec1 transformant was plated on the surface of agar, and colonies were transferred with toothpicks to plates with

Table 1. Postreplication repair of BC200rec1 with and without pMuc

Presence of pMuc	UV dose, J/m²	Time of incubation after pulse label, min	Weight average $M_r \times 10^{-8}$
+	0	40	1.4
+	2.5	0	0.8
+	2.5	20	1.2
+	2.5	40	1.3
−	0.8	0	0.6
−	0.8	40	0.8

UV doses were adjusted to yield comparable survival for the two strains.

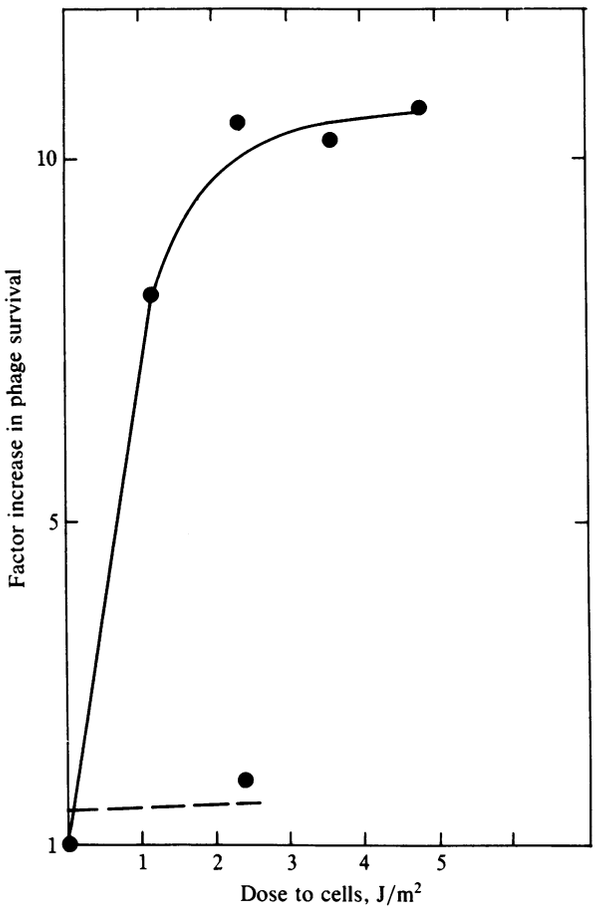

FIG. 4. Survival of HP1*c*1 phage irradiated with a UV dose of 144 J/m² as a function of dose to BC200rec1 host cells with (—) and without (---) pMuc. Survival of phage in unirradiated cells was 0.002%.

and without chloramphenicol, resistance to which was coded for by the plasmid. Two of 260 colonies screened had become chloramphenicol sensitive, indicating that they had lost the plasmid. Gel electrophoresis of cleared lysates of the two clones confirmed the loss of the plasmids. The frequency of spontaneous mutation to novobiocin resistance was now at the same low level as that of the BC200rec1 before transformation, and there was no longer UV-induced mutation. However, the survival after UV was the same as that of the strain with the plasmid, and Weigle reactivation was also unchanged (data not shown). Similarly, wild-type Rd(pMuc) also lost the high spontaneous mutation to novobiocin resistance when it lost the plasmid.

The two transformed strains of BC200rec1 that had lost the plasmid regained the high spontaneous mutation upon retransformation with pMuc.

pMuc in Another *rec1* Mutant Strain. When pMuc from an original BC200rec1 transformant was put into another *rec1* mutant strain (the same allele), there was no effect on UV resistance and spontaneous or UV-induced mutation (data not shown).

Complementation of *mucA*⁻ But Not *mucB*⁻ by pMuc. The doses at which $1/e$ of the initial population survived after UV-irradiation, obtained from the data for TK610, TK610 (pGW1700mucA123:Tn1000)(pMuc), and TK610(pGW1700-mucB58:Tn1000)(pMuc), were 0.56, 1.0, and 0.56 J/m², respectively, and UV-induced mutagenesis was only observed for TK610(pGW1700mucA123:Tn1000)(pMuc), indicating that pMuc could substitute for a deficient *mucA* gene in enabling the pKM101 genes to overcome the *umuC* mutation of TK610.

Hybridization of pMuc and Chromosomal DNA with Probes Containing *muc* Genes. pMuc hybridized with pSE200, which contains all of *mucA* but only a very small portion of *mucB*, and also with pGW1700, which contains both the *mucA* and *mucB* genes intact. Hybridization was observed of pGW1700 but not pSE200 with the chromosomal DNA of the cured BC200rec1 strain, indicating that at least part of the *mucB* gene had entered the *H. influenzae* chromosome.

DISCUSSION

We have shown that pMuc causes UV-induced and high spontaneous mutation in strains of *H. influenzae* proficient in postreplication repair. The UV resistance, postreplication repair capability, and Weigle reactivation of the original BC200rec1 transformed by the ligation mixture was retained when pMuc was lost. Therefore, it is probable that there had been recombination of some of the 7.8-kbp fragment from pKM101 into the chromosome during the original transformation of BC200rec1 by that fragment ligated to the vector. Hybridization data, complementation, and restriction analysis of pMuc indicated that only *mucA* could be on the plasmid. Thus, we conclude that *mucB* on the chromosome of BC200rec1 caused the phenotypic changes in that strain, in accord with the hybridization of DNA of the cured BC200rec1, with the *mucAmucB* probe, but not with the *mucA* probe, lacking most of *mucB*. The *mucA* gene altered the phenotype of only the *rec1* mutant strains whose chromosome contained the *mucB* gene but did alter the phenotype of all of the strains proficient in postreplication repair, suggesting that the latter strains already contain an analog of *mucB* on their chromosome. However, the *mucB* gene or its putative analog on the *H. influenzae* chromosome can be expressed without the presence of the *mucA* gene, as indicated by the properties of BC200rec1 carrying *mucB* but not *mucA*.

Although the functions of *mucA* and *mucB* genes have been separated in *H. influenzae*, these functions are not obviously distinguishable in *E. coli*, since a mutation in either *mucA* or *mucB* of pKM101 abolishes the ability of the plasmid to cause UV-induced mutation or increased UV resistance in *umuC* and *umuD* mutant strains of *E. coli* (17). Another difference between the effect of pKM101 genes on *E. coli* and *H. influenzae* is that pKM101 does not exert its effect in *recA* mutant strains of *E. coli* (2, 3, 30). In some respects—UV sensitivity, noninducibility of prophage, and DNA degradation—BC200rec1 of *H. influenzae* is similar to the *recA* mutant strain of *E. coli* (31). However, the *H. influenzae* mutant was clearly different from *recA* mutant strain of *E. coli* in its response to pKM101 genes. It became resistant to UV and proficient in postreplication repair but not proficient in recombination because of one of these genes, whereas the *recA* mutant strain is unchanged by these genes. One possible explanation for some of these phenomena might be that BC200rec1 is a double mutant. However, there is strong evidence that the recombination defect of the mutant is the same mutation as the defect in UV repair (12).

There are several puzzling aspects of our results. The Umu$^-$ *E. coli* mutant performs postreplication repair as efficiently as does the corresponding wild type (32), but postreplication repair capability was acquired in *H. influenzae* BC200rec1 from the *mucB* gene. The gene from pKM101 apparently entered the chromosome of BC200rec1 in all 17 clones in which the hybrid plasmid was established, in spite of the fact that the transformation frequency of these mutants is 10^{-6} of that of the wild type (12). These data suggested that the pKM101 gene may have become integrated into the chromosome by a mechanism different from the usual recombination of transformation, such as by some kind of transposition. There are inverted repeats around the *muc* genes of pKM101, but the *Hpa* I fragment used for our cloning should have included only one of them, according to the published restriction maps (25, 33).

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1. Walker, G. C. (1984) *Microbiol. Rev.* **48,** 60–93.
2. Walker, G. C. & Dobson, P. P. (1979) *Mol. Gen. Genet.* **172,** 17–24.
3. Walker, G. C. (1977) *Mol. Gen. Genet.* **152,** 93–103.
4. Kato, T. & Shinoura, Y. (1977) *Mol. Gen. Genet.* **156,** 121–131.
5. Shinagawa, H., Kato, T., Ise, T., Makino, K. & Nakata, A. (1983) *Gene* **23,** 167–174.
6. Elledge, S. J. & Walker, G. C. (1983) *J. Mol. Biol.* **164,** 175–192.
7. Boling, M. E. & Kimball, R. F. (1976) *Mutat. Res.* **37,** 1–10.
8. Kimball, R. F. & Hirsch, B. F. (1975) *Mutat. Res.* **30,** 9–30.
9. Walker, G. C., Elledge, S. J., Kenyon, C. J., Kreuger, J. H. & Perry, K. L. (1982) *Biochimie* **64,** 607–610.
10. Balganesh, M. & Setlow, J. K. (1984) *Mutat. Res.* **125,** 15–22.
11. Barnhart, B. J. & Cox, S. H. (1968) *J. Bacteriol.* **96,** 280–282.
12. Setlow, J. K., Boling, M. E., Beattie, K. L. & Kimball, R. F. (1972) *J. Mol. Biol.* **68,** 361–378.
13. LeClerc, J. E. & Setlow, J. K. (1973) *Nature (London) New Biol.* **241,** 172–174.
14. McCann, J., Spingarn, N. E., Kobori, J. & Ames, B. N. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 979–983.
15. Mortelmans, K. E. & Stocker, B. A. D. (1979) *Mol. Gen. Genet.* **167,** 317–327.
16. McCarthy, D., Clayton, N.-L. & Setlow, J. K. (1982) *J. Bacteriol.* **151,** 1605–1607.
17. Perry, K. L. & Walker, G. C. (1982) *Nature (London)* **300,** 278–281.
18. Elledge, S. J. & Walker, G. C. (1983) *J. Bacteriol.* **155,** 1306–1315.
19. Harm, W. & Rupert, C. S. (1963) *Z. Vererbungsl.* **94,** 336–348.
20. Notani, N. K., Setlow, J. K., McCarthy, D. & Clayton, N.-L. (1981) *J. Bacteriol.* **148,** 812–816.
21. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
22. Steinhart, W. L. & Herriott, R. M. (1968) *J. Bacteriol.* **96,** 1718–1724.
23. Schleif, R. F. & Wensink, P. C. (1981) *Practical Methods in Molecular Biology* (Springer, New York).
24. McDonell, M. W., Simon, M. N. & Studier, F. W. (1977) *J. Mol. Biol.* **110,** 119–146.
25. Langer, P. J., Shanabruch, W. G. & Walker, G. C. (1981) *J. Bacteriol.* **145,** 1310–1316.
26. Setlow, J. K., Brown, D. C., Boling, M. E., Mattingly, A. & Gordon, M. P. (1968) *J. Bacteriol.* **95,** 546–558.
27. Notani, N. K. & Setlow, J. K. (1980) *J. Bacteriol.* **143,** 516–519.
28. LeClerc, J. E. & Setlow, J. K. (1972) *J. Bacteriol.* **110,** 930–934.
29. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
30. Waleh, N. S. & Stocker, B. A. D. (1979) *J. Bacteriol.* **137,** 830–838.
31. Kooistra, J. & Setlow, J. K. (1976) *J. Bacteriol.* **127,** 327–333.
32. Kato, T. (1977) *Mol. Gen. Genet.* **156,** 115–120.
33. Winans, S. C. & Walker, G. C. (1985) *J. Bacteriol.* **161,** 402–410.